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Purification and Properties of a Factor From Insect Hemolymph That Promotes Multicellular Vesicle Formation In Vitro

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A vesicle promoting factor (VPF) in an insect cell line IAL-TND1 was partially purified from larval hemolymph of the cabbage looper, *Trichoplusia ni* Hübner. The polypeptide had a molecular weight estimated to be between 20.5 kD and 37.5 kD by gel permeation, 22.5 kD by the Ferguson plot on nondenaturing polyacrylamide gel electrophoresis, and 16.88 kD on sodium dodecyl sulfate PAGE. VPF fractions were isolated from hemolymph by gel permeation on Fractogel® and were either subjected to chromatofocusing or preparative isoelectric focusing. After the gel permeation step, the VPF polypeptide was highly unstable during the separation and storage. The two active fractions from the isoelectric separations had isoelectric points of 6.21 and 6.36 and had specific activities of 34 and 32 vesicles/μg protein per test culture chamber. The percentage of total larval equivalent hemolymph proteins found in these fractions was less than 1%. Chromatofocusing technique also yielded an active fraction containing a single band on nondissociating electrophoresis that had VPF activity. This band had an isoelectric point of 6.60 but had a lower specific activity of three vesicles/μg protein in the VPF cell bioassay.

Key words: polypeptide, hemolymph, cell line, multicellular vesicle

INTRODUCTION

Establishment of insect cell lines provides defined systems for fundamental studies on insect development. The primary processes crucial for morphogenesis are cell division, migration, cell recognition and adhesion, differentiation, and cell death [1]. Lynn and Oberlander [2] developed a cell line from imaginal wing discs of the cabbage looper, *Trichoplusia ni* Hübner,

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which facilitates studies on morphogenesis of insect cells. These cells grew originally as multicellular vesicles, but after a year underwent a morphological change in culture to an aggregate (clumped) culture. Lynn et al. [3] recently reported that a reversal to the vesicle form could be induced by the addition of larval hemolymph to the aggregate cultures. Vesicle-promoting activity of the hemolymph was found to be heat-sensitive and protease-sensitive. We report evidence that the active fraction in hemolymph of *T. ni* is a protein that causes the aggregates to form multicellular vesicles. The protein has been partially purified and some of its properties are described.

MATERIALS AND METHODS

Hemolymph Collection and Treatment

Hemolymph was collected from prewandering fifth-instar larvae (weight 280–300 mg/larva) by cutting a proleg and collecting the fluid with a 20-μl capillary tube. To reduce melanization, the hemolymph was immediately added to a test tube on ice that contained 0.06% cysteine and 0.3 M sucrose in 50 mM Tris-HCl, pH 7.5. Hemolymph was collected until the concentration was 50% of hemolymph in buffer, and then the sample was centrifuged (13,000 *g*, 5 min) to remove cellular material. Samples were stored at −69°C until applied to a gel permeation column.

Gel Permeation, Isoelectric Focusing, and Chromatofocusing of Hemolymph

Four milliliters of 50% hemolymph were applied to a Fracto-gel® (TSK HW-55, 32–63 μm, fractionation range of 1–700 kD, Pierce Chemical Co., Rockford, IL) column (2.5 × 53.5 cm) that was equilibrated with 50 mM Tris-HCl, pH 7.5, containing 20 mM NaCl and 0.03% cysteine at 4°C. The flow rate was 30 ml/h, and 5 ml fractions were collected. The fractions were concentrated to 2.0 ml against Ringer's solution [4], using a Pro Di Mem® (10,000 molecular weight) concentrator and assayed. To determine the molecular weight of the hemolymph proteins, a calibration curve was prepared using the following protein standards (30 mg) from Pharmacia: aldolase (158 kD); bovine serum albumin (67 kD); chymotrypsinogen A (25 kD); and ribonuclease A (13.7 kD). After the molecular weight of the VPF protein was estimated on this column, hemolymph (2.0 ml of 50% hemolymph) was run on a Fracto-gel® (TSK HW 50F, 32–63 μm, fractionation range 0.5–80 kD, Pierce Chemical Co., Rockford, IL) column (6 × 96 cm) that was equilibrated with 50 mM Tris-HCl, pH 7.5, containing 20 nM NaCl and 0.03% cysteine at 4°C. The flow rate was 30.4 ml/hr and 5 ml fractions were collected. Molecular weight standards were from Sigma (St. Louis, MO): aprotinin (6.5 kD) 3.5 mg; cytochrome (12.4 kD) 3.5 mg; carbonic anhydrase (29 kD) 3.5 mg; and bovine serum albumin (66 kD) 8 mg.

The concentrated fraction containing VPF (10.5 mg protein from three runs on Fracto-gel®, 6 ml total hemolymph) was then subjected to preparative flat-

Abbreviations: kD = kilodalton; PBE = polybuffer exchanger 94®; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; VPF = vesicle-promoting factor.

bed isoelectric focusing using Sephadex G-75 (Pharmacia, Piscataway, NJ) as stabilizing medium (LKB 2117 Multiphor, 2% ampholyte, pH 5–7 (LKB, Gaithersburg, MD) [5]. The separated zones were collected after sectioning the gel bed into 28 fractions and the pH of alternates measured. Proteins were eluted from each gel fraction with 10 ml 2× Ringer's solution and dialyzed and concentrated against 1× Ringer's to 1 ml using the Pro Di Mem® unit.

Protein content was assayed according to Bradford [6], using bovine gamma globulins as the standard.

Fractions from gel permeation that contained VPF were also applied to a chromatofocusing column (1 × 30 cm) containing PBE 94® [7]. Proteins were eluted according to their isoelectric points using Polybuffer 74-HCl ® (pH range 7–5). Prior to sample application, the column was equilibrated with 2 liters of start buffer, 25 mM imidazole, pH 7.5. The column was then washed with Polybuffer 74-HCl® (diluted 1:6 with H_2O), pH 5.0, at a flow rate of 7.2 ml/h; 1.2 ml fractions were collected.

A concentrated sample (19 mg protein), which contained VPF, obtained from separation of hemolymph on Fracto-gel®, was dissolved in 4.2 ml of start buffer and applied to the chromatofocusing column at 4°C.

To remove polybuffer, which apparently can form aggregates up to 20,000 molecular weight units, the fractions containing UV absorbing peaks at 280 nm were combined and applied to a Sephadex G-75 column (0.9 × 60 cm) and eluted with 50 mM Tris-HCl, pH 7.5, at 18 ml/h. The resultant fractions absorbing at 280 nm were combined, lyophilized, and dissolved in 1.2 ml Ringer's solution. This solution was then filter-sterilized using a 0.2 μm Nalge® nylon filter.

Cell Cultures and Assay

The cell line IAL-TND1, developed from imaginal discs of *T. ni*, was used to assay vesicle-promoting activity of hemolymph and hemolymph fractions [2,3]. Generally, the sample in Ringer's solution was filter-sterilized, and 200 μl was added to 1.8 ml of Grace's media that also contained 0.03% cysteine. Then 250 μl of this stock solution was added to the IAL-TND1 cells in multiwell plates. Each well contained 250 μl of Grace's medium plus 0.03% cysteine. The concentration of protein in the samples tested ranged from 500 to 600 μg of protein in whole hemolymph and from 15 to 60 μg in the fractions. Each well contained approximately 1×10^4 cells in 26-well plates. Plates were maintained at 26°C and vesicles were counted after 6 days of exposure to test media. Specific activity refers to the number of vesicles in a test well minus the number in the control well adjusted to a μg-protein basis.

Electrophoresis

SDS-PAGE was carried out using the Bio-Rad Protean I® vertical cell and the Model 360 minivertical Slab-Cell®, according to the dissociating conditions described by Swanton et al [8]. Molecular weight standards were from Pharmacia. A calibration curve was prepared using molecular weight standards from LKB: phosphoylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), and ferritin (18.5 kD). The relative migra-

tion of each of the bands was plotted against the log of the molecular weight. Nondissociating electrophoresis was carried out according to Davis [9]. The Ferguson plot was used to determine the molecular weights of the protein in nondissociating gels [10]. Protein molecular weight standards were from Sigma (St. Louis, MO). The active VPF fraction, after purification by isoelectric focusing, was run on 4.5, 5.5, 5, 6.10, 7, 8, and 9% polyacrylamide tube gels. Thirty-five micrograms of VPF protein was applied to 5, 6, 7, and 8% gels. The following protein standards were run on the following percentage of polyacrylamide: α-lactalbumin, 7, 8, 9, 10%; carbonic anhydrase 6, 7, 8, 9%; chicken egg albumin, 7, 8, 9, 10%; and bovine serum albumin 7, 8, 9, 10 and 4.5, 5, 5.5, 6%. The Rf of each of the sample proteins and standards in each gel relative to the tracking dye were plotted against the percent gel concentration. The logarithm of the negative slope obtained from this plot was then plotted against the logarithm of the molecular weight of each protein to produce a linear plot from which the unknown molecular weights were determined. The gels were stained with Coomassie blue R-250® or silver-stained using the procedure of Wray et al. [10].

RESULTS

Gel Permeation

Figure 1 shows the elution profile of hemolymph separated on Fracto-gel. Recovery of whole hemolymph protein (minus 13,000 *g* pellet) run on the column was 97.1% (185.6 mg applied; 180.3 mg recovered). Vesicle-promoting activity was detected in only two 5-ml fractions that eluted (182 ml to 192 ml) between the two molecular weight standards, ovalbumin (43 kD) and ribonuclease A (13.7 kD). Chymotrypsinogen A (25 kD) eluted within the area of VPF activity, indicating that the molecular weight of the VPF was close to that of this standard. The fractions containing the VPF activity and two fractions on each side bracketing this activity were combined (6.1 mg protein;

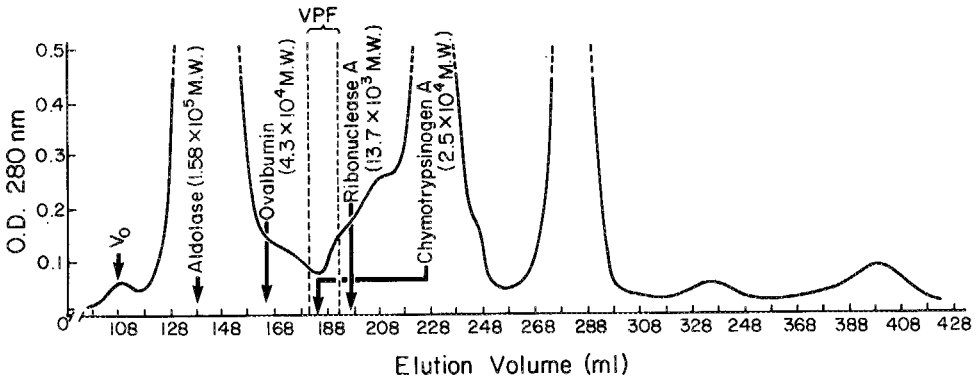

Fig. 1. Molecular-sieve chromatography of cabbage looper larval hemolymph on Fracto-gel®. A sample containing 185 mg of hemolymph protein was applied to the column (2.5 × 53.5 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 20 mM NaCl and 0.03% cysteine. Flow rate was 30 ml/h, and 5-ml fractions were collected. VPF indicates tubes that contain vesicle-promoting activity in cell bioassay. Thirty milligrams of each molecular weight standard were applied.

3.3% of total recovered protein) and run on Fracto-gel® with a narrower fractionation range (0.5–80 kD). Figure 2 shows the calibration curve used to estimate the molecular weight of VPF. Because VPF activity eluted in five fractions, the estimated molecular weight ranged from 20.5 to 37.5 kD.

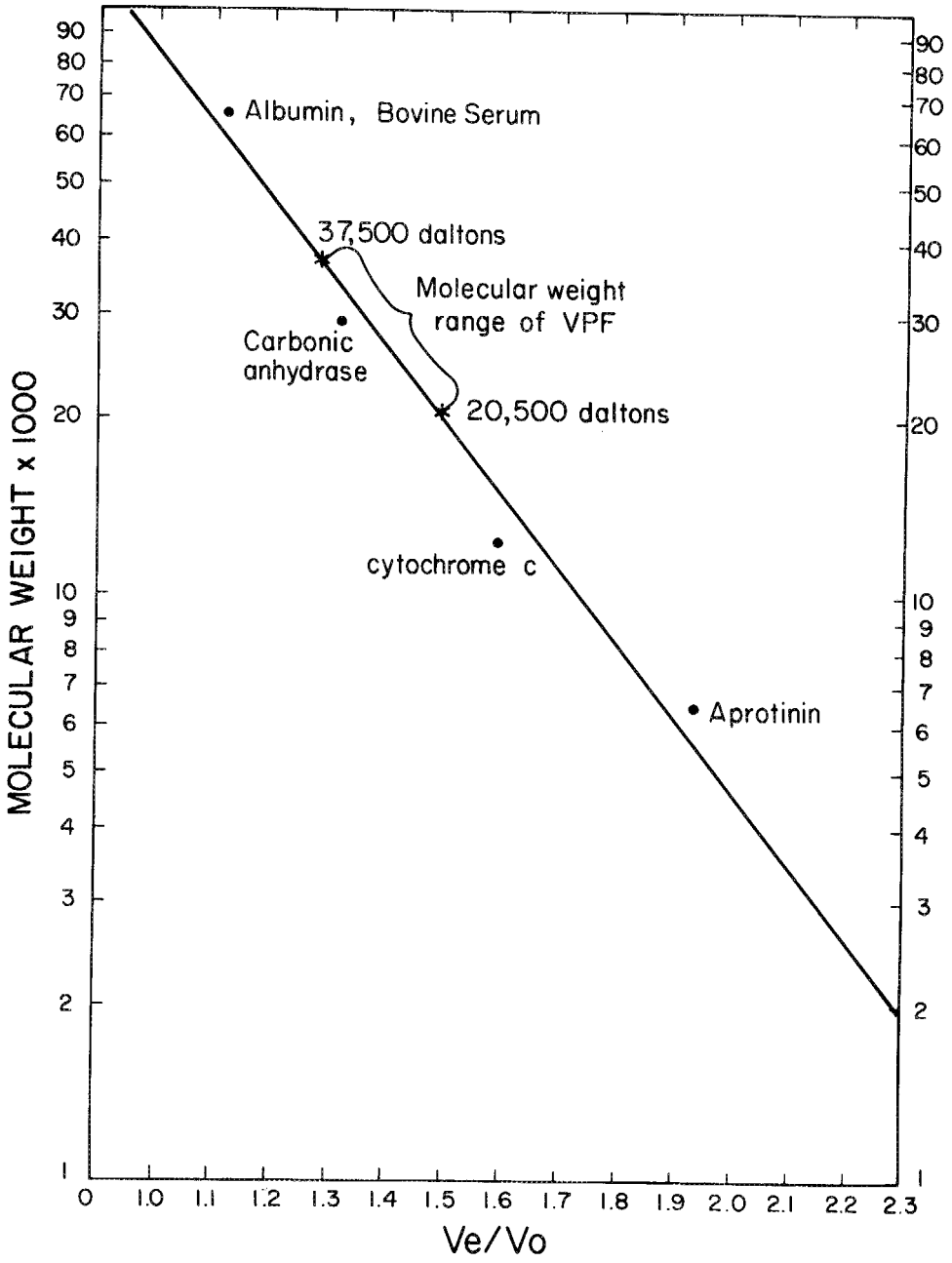

Fig. 2. Calibration curve for estimation of the molecular weight of VPF by gel permeation. Ve/Vo.

Preparative Isoelectric Focusing

Alternate fractions from the 28 that resulted from the isoelectric focusing separation were bioassayed for VPF activity. The quantity of protein in the fractions is shown in Figure 3A. Of the total amount of protein from gel permeation applied (10.5 mg) in the isoelectric separation, 5.9 mg, or 56.3% was recovered. Of the 5.9 mg protein recovered, 0.94 mg and 0.36 mg were recovered in fractions 8 and 9, respectively, which had VPF activity. Fractions 8 and 9 had isoelectric points of 6.21 and 6.36, respectively, and had a specific activity of 34 vesicles/μg protein and 32 vesicles/μg protein, respectively (Fig. 3B). The percentage of total hemolymph protein associated with VPF activity in these experiments was 0.74%. On a nondissociating polyacrylamide gel, fractions contained a wide Coomassie blue staining band that stained heavier in fraction 8 than in 9 and had an Rf of 0.41 (Fig. 3C, lanes 2 and 3, labeled b). A slower migrating band (Rf = 0.1) also was present in fractions 8 and 9 as well as in fraction 7. Fraction 7 had little of the fast migrating band and did not have VPF activity (see Fig. 3B and lanes 1 of Fig. 3C, labeled a). When fractions 8 and 9 (shown in lanes 2 and 3 in Fig. 3C) were run on an SDS-dissociating polyacrylamide gel, the most evident subunits, going down the gel, were 108, 663, 73, 685, and 16,880 daltons (Fig. 3D, lanes 2 and 3). This latter 16.88 kD band was the broadest and heaviest staining subunit.

When the slow migrating band was cut out of a nondissociating gel (Fig. 4, lane 1, labeled a) and run on an SDS-dissociating gel, only a 73-kD band was apparent (Fig. 4, SDS-PAGE, lanes 2, 4, and 6, labeled a). When the broad, faster running band was cut out of a dissociating gel and run on SDS-PAGE, only a 16.88-kD subunit was evident (lanes 3 and 5, labeled b).

The molecular weight of proteins in the active VPF fraction obtained by isoelectric focusing was also estimated on nondissociating gels using the Ferguson plot, as shown in Figure 5. The apparent molecular weight of the broad band observed in the nondissociating gel (Fig. 3C, lanes 2 and 3, labeled b) had a molecular weight of 22.5 kD by this method.

Chromatofocusing

Figure 6A shows the elution profile of VPF protein on a chromatofocusing column after applying pooled active fractions from gel permeation. Of the 19 mg of protein from gel permeation applied, 5.76 mg was recovered in tubes 44 and 45. These fractions also contained VPF activity (Fig. 6B). The pH of each of these fractions was 6.61 and 6.60, respectively. After running these fractions on a gel permeation column to remove the Polybuffer®, the protein was highly unstable and easily precipitated from solution. Only 0.3% of the protein was recovered at this stage of purification. The specific activity of this fraction was 3 vesicles/μg protein, considerably less than that obtained with the isoelectric focusing preparation.

Nondissociating electrophoresis on the minivertical slab gels carried out immediately after isolation of the VPF protein showed that the protein was present as a single band on a silver-stained gel with an Rf of 0.4 (Fig. 6C) and had an Rf similar to that for protein "b" (Rf = 0.41) in Figure 3C.

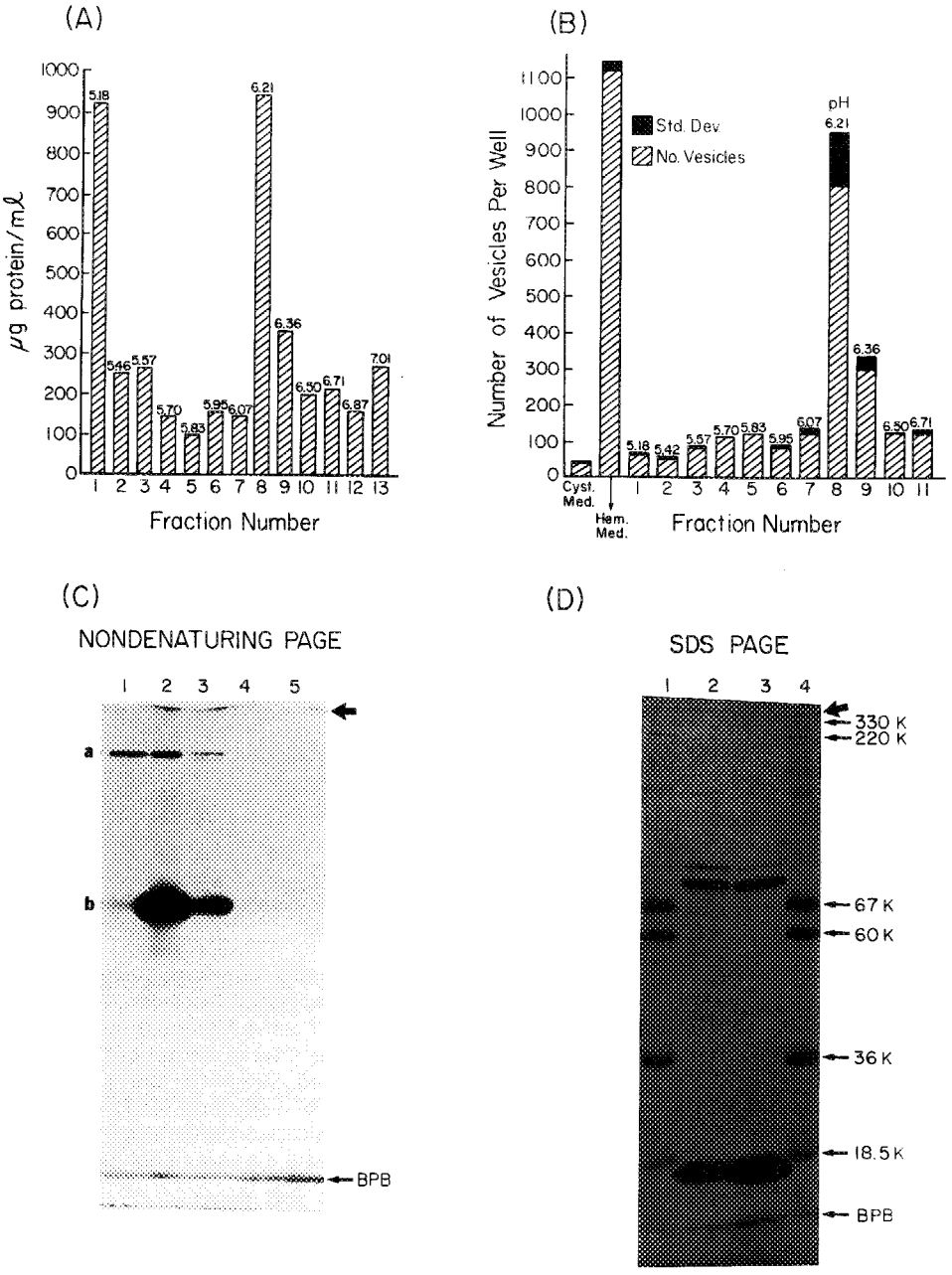

Fig. 3. Isoelectric focusing of combined fractions that contain VPF from molecular-sieve chromatography of cabbage looper larval hemolymph. A pH gradient of 5 to 7 was developed in Sephadex G-75. **A)** Protein content and pH of fractions. **B)** VPF activity and pH of fractions. **C)** Nondenaturing PAGE of fractions 7, 8, 9, 10, and 11 in lanes 1, 2, 3, 4, and 5, respectively. BPB, bromophenol blue marker. **D)** SDS PAGE of fractions 8 and 9 in lanes 2 and 3. Molecular weight standards in lanes 1 and 4: thyroglobulin (330 kD), ferritin (220 kD), albumin (67 kD), catalase (60 kD), lactate dehydrogenase (36 kD), ferritin subunit (18.5 kD) and BPB. The correlation coefficient (0.98) for the plot of log of the molecular weight of the standards vs. Rf was highly significant at the 99% level. The concentration of the running gel was 12.5%. Heavy arrows in C and D indicate surface of separating gel. Gels were stained with Coomassie blue.

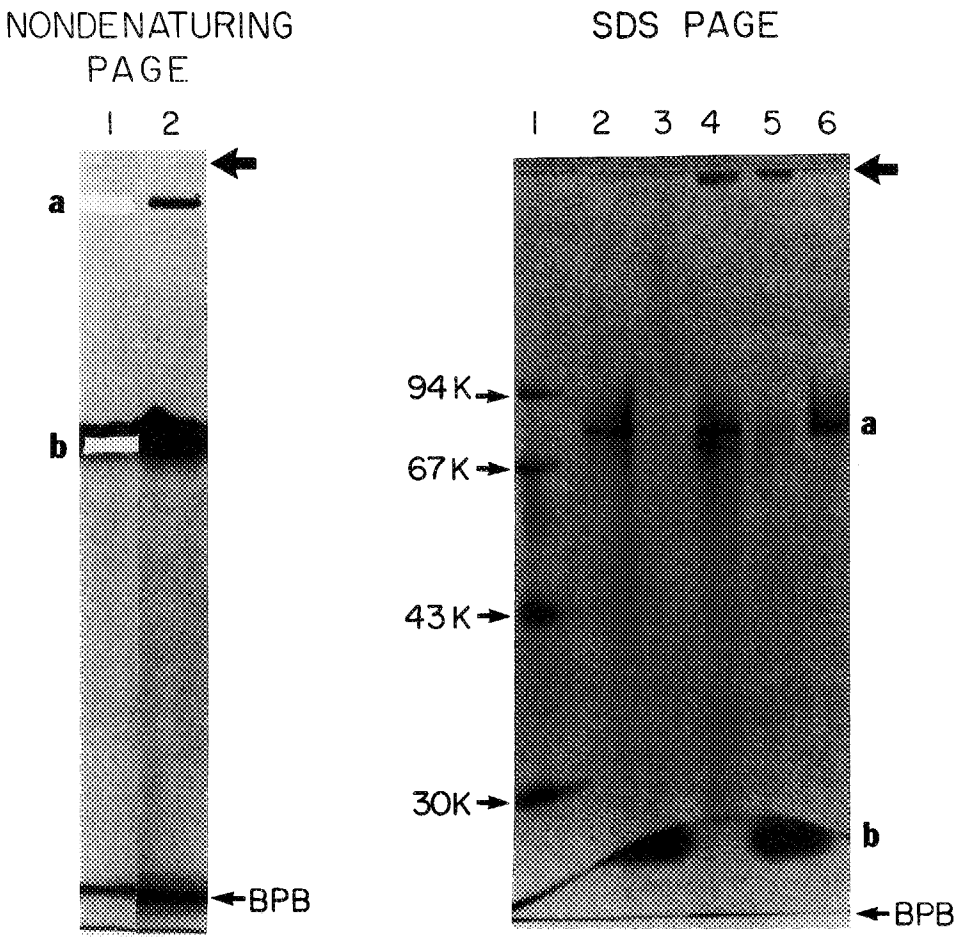

Fig. 4. Protein bands from a VPF active fraction cut out of nondenaturing polyacrylamide gel and run on SDS PAGE. The VPF fraction was isolated by preparative isoelectric focusing as described in Figure 2. Nondenaturing PAGE: lanes 1 and 2, VPF active fraction from isoelectric focusing separation, isoelectric point 6.21. The bands in lane 1 were cut out of the gel and placed on the surface of the SDS gel and then electrophoresed. SDS PAGE: lane 1, molecular weight standards: phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD); lanes 2, 4, and 6—band labeled a from lane 1, nondenaturing PAGE; lanes 3 and 5—band labeled b from lane 1, nondenaturing PAGE. BPB, bromophenol blue marker, stained with Coomassie blue.

DISCUSSION

These results indicate that a polypeptide present in hemolymph of the cabbage looper moth, *T. ni*, induces multicellular vesicle formation in an insect cell line. Our procedure for purifying the factor involved running larval hemolymph plasma first on a molecular sieve column, bioassaying the resultant fractions for cell vesicle-promoting activity, and then purifying the active fraction on a preparative flat-bed isoelectric focusing unit or on a chromatofocusing column. Estimation of the molecular weight of the VPF by gel permeation indicated that it was between 20.5 kD and 37.5 kD. Based on

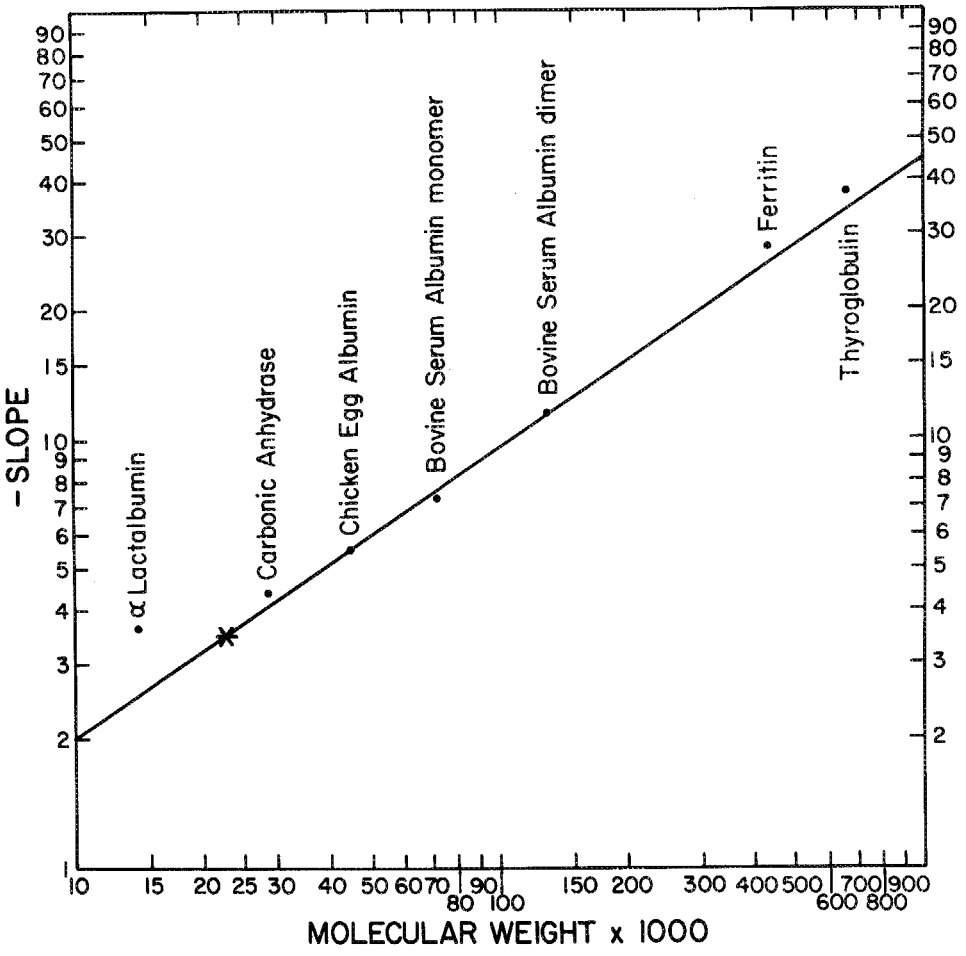

Fig. 5. Calibration curve for determining the molecular weight of the VPF on nondenaturing gels.

results obtained from the isoelectric technique, the active fractions contained a major band with a molecular weight of about 22.5 kD on nondissociating polyacrylamide gels and 16.88 kD on SDS polyacrylamide gels. The only active fraction from the chromatofocusing run contained a broad staining band with an Rf of 0.41 on a 7.5% nondissociative gel and thus had an Rf value similar to the major band (Rf 0.41) in the two active isoelectric focusing fractions on a gel of the same concentration. However, there were differences in the isoelectric points. The isoelectric point of the chromatofocusing fraction was 6.6, and was higher than the isoelectric points of the two active fractions obtained by isoelectric focusing, 6.21 and 6.36. These differences could be explained by the difficulty we had running the hemolymph samples on the chromatofocusing columns. Although the chromatofocusing technique resulted in a fraction of greater purity than the isoelectric technique, it was also subject to variation in results due to the instability of the purified protein in

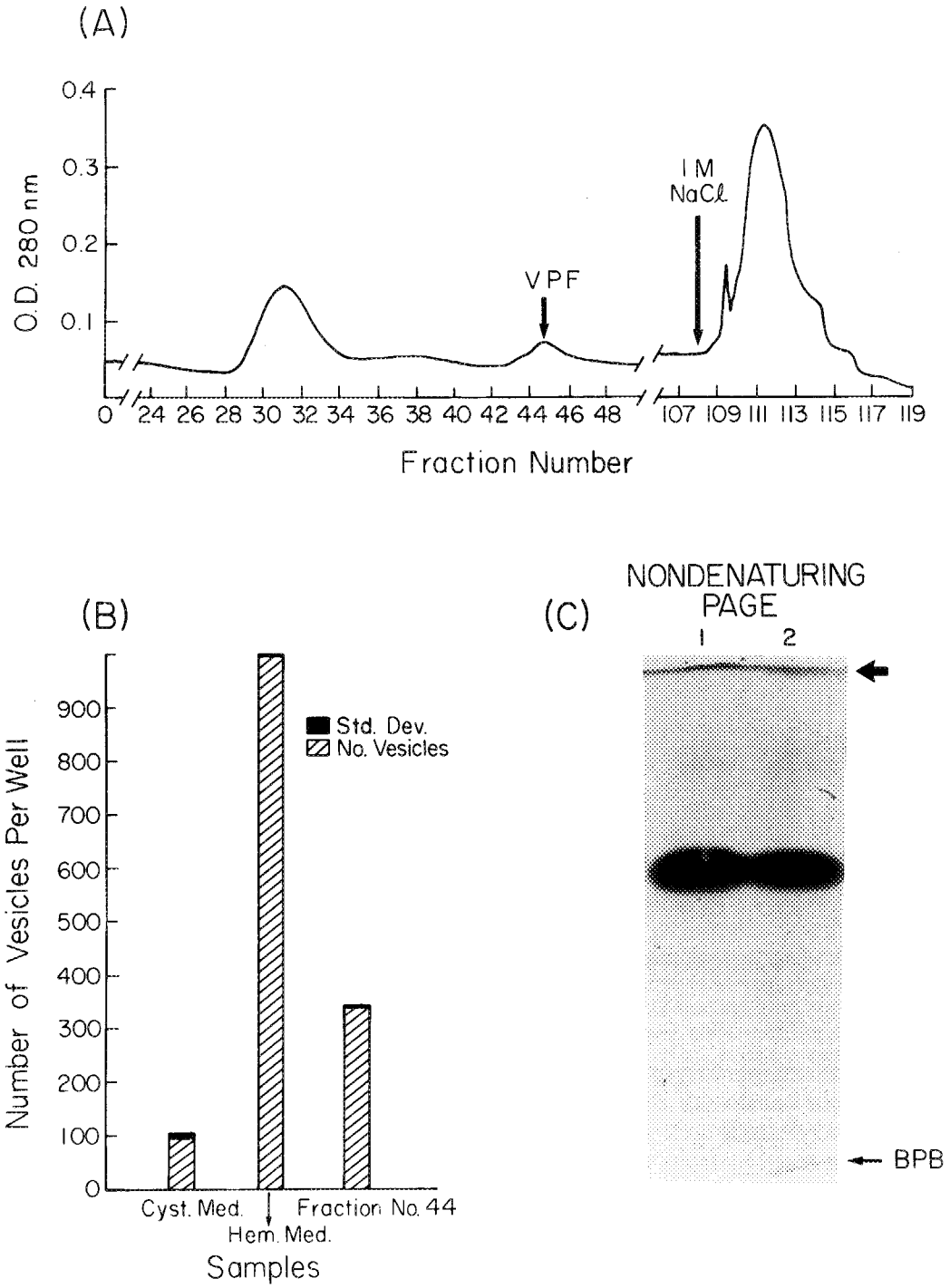

Fig. 6. Chromatofocusing of pooled fractions that contain VPF from molecular-sieve chromatography of cabbage looper hemolymph. **A)** Elution of protein on a pH gradient of 5–7; NaCl wash removed remaining proteins. VPF indicates fractions that contain vesicle-promoting activity (nos. 44 and 45). **B)** VPF activity in combined fraction numbers 44 and 45 (labeled no. 44) in the figure). **C)** Nondenaturing PAGE of combined fraction numbers 44 and 45. BPB, bromophenol blue marker. Heavy arrow indicates the surface of separating gel. Stained with silver stain. Cyst. Med., cysteine medium; Hem. Med., hemolymph medium.

solution. Therefore, the true isoelectric point of the VPF probably is closer to that obtained by isoelectric focusing.

The VPF polypeptide was found to be a highly unstable protein, especially after it was purified by either isoelectric focusing or chromatofocusing, and would readily form aggregates, which we observed on nondissociating PAGE and during separation by gel permeation (data not shown). This tendency of the VPF to form aggregates may be the reason for the higher molecular weight of > 60 kD obtained by dialysis and reported earlier [3]. Examination of the VPF active fractions from isoelectric focusing runs on SDS-PAGE indicated that the protein was not purified to homogeneity. There were also faint minor bands evident in addition to the major, heavy-staining, 16.88-kD band, and narrow, lighter-staining, 73,000, and 108,663-dalton bands. One of these minor bands may be responsible for the VPF activity observed in our bioassays. However, in 13 preparative isoelectric focusing runs, only when we observed the broad, heavy-staining band (Rf 0.41) on 7.5% nondissociating PAGE did we also observe VPF activity in the fraction (as in lanes 2 and 3, Fig. 3C). When this band was absent (as in lane 1, Fig. 3C), VPF activity was also absent. Therefore, from this correlation we conclude that the low-molecular-weight band that has an estimated molecular weight of 16.88 kD on SDS PAGE and 22.5 kD on nondissociating PAGE is the major polypeptide responsible for VPF activity.

Further purification of the VPF is under way, as well as studies directed toward understanding the mechanism of action of VPF at the cellular level. Whether the morphogenic effect of VPF in vitro reflects a related role in vivo remains to be determined.

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